INTERLABORATORY COMPARISON OF CHRONIC TOXICITY TESTING USING THE ESTUARINE MYSID (MYSIDOPSIS BAHIA): A FINAL REPORT

CHARLES L. MCKENNEY, JR.

U.S. ENVIRONMENTAL PROTECTION AGENCY

ENVIRONMENTAL RESEARCH LABORATORY

GULF BREEZE, FLORIDA 32561

(Author Comm Used)

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL RESEARCH LABORATORY
SABINE ISLAND
GULF BREEZE, FLORIDA 32561

March 12, 1982

SUBJECT: Final Report for the Interlaboratory Comparison of Chronic Toxicity Testing Using the Estuarine Mysid (Mysidopsis bahia)

FROM: Charles L. McKenney, Jr.
Research Aquatic Biologist
Experimental Environments Branch

TO: Steven Ells
Health and Environmental Review Division
Office of Toxic Substances (TS-796)

Attached is the Final Report on the "Interlaboratory Comparison of Chronic Toxicity Testing Using the Estuarine Mysid (Mysidopsis bahia)." In this report laboratories are coded by number. The key to this code is as follows: Laboratory 1 is ERL, Gulf Breeze, FL; Laboratory 2 is ERL, Narragansett, RI; Laboratory 3 is Jones, Edmunds and Associates, Gainesville, FL; Laboratory 4 is EG&G Bionomics, Pensacola, FL (subcontracted by Southwest Research Institute, Houston, TX); Laboratory 5 is Battelle's William F. Clapp Laboratory, Duxbury, MA; and Laboratory 6 is New England Aquarium, Boston, MA.

As a result of this project, a number of modifications were recommended for use in the chronic toxicity test using M. bahia. Over the past months, these modifications have been successfully adopted by our laboratory for use in our chronic mysid toxicity testing with a number of pesticides. As suggested in the final report, these modifications have resulted in a more powerful test with a reduction in response variation. This modified method has been incorporated in a manuscript that is in press for publication, is discussed in an annual report that is in preparation, and will be included in the revised ASTM draft for "Chronic Toxicity Testing with Mysids" by myself as acting co-chairman of this ASTM Task Group.

If there are any questions regarding this final report or the revised chronic toxicity test method with mysids, please don't hesitate in contacting me.

Attachment

INTRODUCTION

The Environmental Protection Agency's Office of Pesticides and Toxic Substances (OPTS) is required by law to provide manufacturers of new chemical products recommended test methods to determine the toxicity of new chemicals. The Toxic Substance Control Act (TSCA) requires that each permit holder perform his own testing of new chemical products and report the results for regulatory decision making. A standard method for conducting basic toxicity tests with aquatic organisms is desirable as a basis for any testing program required under TSCA. Before proposing a standard method for toxicity testing, OPTS must know the precision of data generated by the test method. Therefore, a series of interlaboratory comparison tests were initiated at the request of OPTS to enable judgement of performance based on data sets gathered under similar conditions.

In 1977, an entire life-cycle toxicity test using the estuarine mysid, Mysidopsis bahia, was introduced (Nimmo et al., 1977). It was suggested that this test method could be used to provide information on the chronic toxicity of chemicals to an ecologically-important estuarine crustacean. This test method has since been used successfully to determine the chronic effects of a heavy metal (Nimmo et al., 1978a) and a number of pesticides (Nimmo et al., 1977; Nimmo et al., 1979; Nimmo et al., 1980; and Nimmo et al., 1981).

The purpose of this project was to provide OPTS information on interlaboratory variation that occurs with this test. Six laboratories were chosen to perform one 28-day life-cycle toxicity test with the heavy metal, silver, and one test with the insecticide endosulfan, using mysids (M. bahia) as test animals. Test chemical selection criteria included the following: toxicity to selected species at or below water solubility, chemical type (an

inorganic and an organic), ease of chemical analysis, and relatively low mammalian toxicity to ensure worker safety.

MATERIALS AND METHODS

Six laboratories were chosen to determine the effects of continuous exposure to silver (as silver nitrate) and endosulfan on the survival and reproduction of Mysidopsis bahia through an entire life cycle. The methods explained in the "Scope of Work" (Appendix) were a modification of the toxicity test for mysids described in "Bioassay Procedures for the Ocean Disposal Permit Program" (Nimmo et al., 1978b).

Test Water

Test water for all laboratories was filtered natural seawater with salinities between 15 and 30 °/oo. Temperatures of test water for five of the six laboratories were specified to be 25 ± 2°C, however, Laboratory 2 used lower test temperatures (22 ± 1°C). In weekly measurements of dissolved oxygen in test water at all laboratories, dissolved oxygen concentrations exceeded 60% saturation.

Exposure System

All laboratories used a flow-through exposure system, employing either infusion pumps for continuous flow of exposure water (Bahner et al., 1975) or diluters for intermittent flow of exposure water (Mount and Brungs, 1967). Flow rates to individual glass exposure aquaria provided no less than 90% replacement in 8 to 12 hours (Sprague, 1969) at all laboratories. Each exposure aquarium for five of the six laboratories contained 8 replicate retention chambers for exposed groups of mysids. Laboratory 2 used only 5 replicate retention chambers. Retention chambers consisted of 10-cm glass

Perti dishes to which 15-cm high cylinders of Nitex[R] screen (210 µm mesh) were attached with silicone cement.

Toxicant Concentrations

A minimum of five concentrations of toxicants and a control-with-carrier was used by all laboratories. Distilled water was the carrier for silver. Concentrations of the carrier for endosulfan (triethylene glycol) did not exceed 5 mg/ℓ in tests by any laboratory. Concentrations of toxicants were based on preliminary 96-hour acute tests; the highest concentration in the life-cycle toxicity test was approximately the 96-hour LC50 value and a dilution factor of 0.5 was used to produce the four lower concentrations. Each week during the test, one water sample from each exposure aquarium was analyzed for toxicant concentrations above the limit of detection. For exposure concentrations below the level of detection, the concentrations of stock solutions used to prepare the non-detectable concentrations were measured.

Test Animals

All test animals were Mysidopsis bahia, as identified by inspection of ten individuals sent by each laboratory to the project officer prior to testing.

Test Initiation

All tests were begun by collecting freshly released juveniles over a 48-hour period from isolated gravid female Mysidopsis bahia. Five laboratories began the test by placing 5 juveniles in each of 8 retention chambers; one laboratory (No. 2) introduced 6 juveniles in 5 retention chambers at the beginning of the test.

[R]Trademark; Tobler, Ernst, and Trabor, Inc., Murray St., New York, NY. Mention of commercial products does not imply endorsement by the U.S. Environmental Protection Agency.

Test Progression

Survival data were recorded daily throughout a 28-day period by all laboratories, except Laboratory 2, which used a test duration of 38 days for the silver test and 39 days for the endosulfan test. Dead mysids were removed daily. Throughout the test, mysids were fed daily 48-hour old Artemia nauplii. As stated in the "Scope of Work", mortality greater than 25 percent among the 40 mysids initially introduced into the control invalidated the test.

On days 10 to 12, or when sex was readily discernible, the numbers of males and females (identified by development of brood pouches) in each retention chamber were recorded. Young released in each retention chamber were counted daily, then removed.

Statistical Analysis

For each retention chamber, reproductive success was determined by dividing the number of young produced by the maximum number of females identified in that chamber. Survival percentages for each retention chamber were calculated by dividing the number of living mysids at the end of 28 days by the initial number of mysids in the chamber less those that died as a result of sticking to the sides of the chamber.

Each laboratory calculated the geometric mean of the MATC (maximum acceptable toxicant concentration) limits obtained from the most sensitive criterion for effects in the life cycle test. The MATC limits ranged between the concentration that had no significant effect on any portion of the life cycle and the lowest concentration that had a significant effect.

Analysis of variance and an appropriate post hoc test (either Student-Newman-Keuls, Duncan's, Dunnett's, or t-tests) were conducted by all

laboratories to compare survival and reproductive success for multiple treatments and controls (USDA Argicultural Research Service, 1977). The level of significance used by all laboratories was 0.05.

To ensure the most objective comparison of the chronic toxicity data from the six laboratories, the raw data from each laboratory were analyzed at the Environmental Research Laboratory, Gulf Breeze, by the same set of statistical procedures: analysis of variance, Dunnett's multiple means comparison procedure, regression analysis, and multiple covariate regression analysis (Steel and Torrie, 1960; Sokal and Rohlf, 1969; Box et al., 1978; and Draper and Smith, 1981).

RESULTS AND DISCUSSION

As stated in the "Scope of Work", the highest concentration to be used in the mysid life cycle toxicity test was to be based on the 96-hour LC50. All laboratories except Laboratory 2 reported the results of 96-hour flow-through toxicity tests with juvenile Mysidopsis bahia (Table 1). The mean 96-hour LC50 for silver is 221 µg/ℓ with a standard deviation of 111 and a 0.50 coefficient of variation. The highest 96-hour LC50 reported for silver was 300 µg/ℓ; the lowest, 64 µg/ℓ. The ratio of the highest LC50 to the lowest LC50 (H/L ratio) is 4.7 and is in close agreement with the H/L ratio of 5.4 for this species, test type, and chemical reported by Schimmel (1981) in an interlaboratory comparison of acute toxicity tests. The mean 96-hour LC50 for endosulfan is 2.62 µg/ℓ with a standard deviation of 2.00 and a 0.76 coefficient of variation. Since the highest 96-hour LC50 for endosulfan was 5.00 and the lowest was 1.05, the H/L ratio for endosulfan is 4.8. As with silver, this H/L ratio for endosulfan closely agrees with the 4.4 value reported by Schimmel (1981) for this species, test type, and chemical as a result of testing by six independent laboratories.

The results from six laboratories of chronic toxicity tests with M. bahia for silver are presented in Appendix Table 1. The MATC limits, MATC geometric means, and effect criteria for silver reported by six laboratories for life cycle toxicity tests with M. bahia are shown on Table 2. Reductions in reproductive success proved to be the most sensitive criterion of effect produced by chronic exposure of M. bahia to silver. The mean of the MATC geometric means for silver that were reported by six laboratories is 32 µg/ℓ with a standard deviation of 31 and a 0.97 coefficient of variation. The highest MATC geometric mean for silver, as reported by the participating laboratories, was 85 µg/ℓ; the lowest value, 5 µg/ℓ. The H/L ratio between laboratories, therefore, is 17. This ratio and the coefficient of variation indicate an unacceptable degree of variation in results obtained among the laboratories using this toxicity test and higher variation than exist for acute toxicity tests with the same species and chemical.

The results of chronic toxicity tests with M. bahia for endosulfan by six laboratories are presented in Appendix Table 2. The MATC limits, MATC geometric means, and effect criteria for endosulfan reported by six laboratories using the mysid life-cycle toxicity test are shown on Table 3. Unlike silver, chronic mortality proved to be the most sensitive criterion of effect for M. bahia exposed to endosulfan. The mean of the MATC geometric means for endosulfan reported by six laboratories is 0.48 µg/ℓ with a standard deviation of 0.29 and a 0.60 coefficient of variation. The highest MATC geometric mean for endosulfan reported by the participating laboratories was 1.00 µg/ℓ; the lowest value was 0.12 µg/ℓ. Therefore, the interlaboratory H/L ratio is an unacceptably high 8.3 and nearly twice that reported for the 96-hour LC50 values, indicating a loss of precision in chronic toxicity values relative to the acute toxicity values reported by the six laboratories.

Table 1. Summary, by laboratory, of the acute toxicity of silver and ensodulfan to juvenile Mysidopsis bahia in flow-through toxicity tests.

Laboratory	96-hour LC 50 (µg/ℓ) Silver	Endosulfan
1	141	1.30
2	_a	_a
3	300	1.05
4	300	5.00
5	64	4.60
6	298	1.14
mean (Geometric mean)	221 (189.1)	2.62
standard deviation	111	2.00
coefficient of variation	0.50	0.76

a- information not provided in final report.

Table 2. MATC limits, MATC geometric means, and effect criteria for silver (µg/ℓ) reported by six laboratories for life cycle toxicity tests with Mysidopsis bahia

Laboratory	MATC Limits (µg/ℓ)	MATC Geometric Mean (µg/ℓ)	Effect Criterion
1	> 9, <25	15	Reproduction
2	>11, <32	19	Reproduction
3	>30, <93	53	Reproduction
4	>14, <19	16	Chronic Mortality
5	>60, <110	85	Chronic Mortality/ Reproduction
6	> 3, <8	5	Chronic Mortality
mean		32	
standard deviation		31	
coefficient of variation		0.97	

Note - All values used in criteria document.
lab 5 published in Dickson et al 1982; 72-110 µg/ℓ
lab 2 published in Hansen et al 1981: 11-32 µg/ℓ

Table 3. MATC limits, MATC geometric means, and effect criteria for endosulfan (µg/ℓ) reported by six laboratories for life cycle toxicity tests with Mysidopsis bahia.

Laboratory	MATC Limits (µg/ℓ)	MATC Geometric Mean (µg/ℓ)	Effect Criterion
1	>0.33, <0.71	0.48	Chronic Mortality/ Reproduction
2	>0.07, <0.19	0.12	Chronic Mortality
3	>0.31, <0.42	0.36	Reproduction
4	>0.28, <0.60	0.41	Chronic Mortality
5	>0.58, <1.71	1.00	Chronic Mortality/ Reproduction
6	>0.43, <0.57	0.50	Chronic Mortality/ Reproduction
mean		0.48	
standard deviation		0.29	
coefficient of variation		0.60	

Laboratory 2 employed a lower test temperature, fewer replicates, and a longer test duration than other laboratories. By eliminating the anomalous chronic toxicity value for endosulfan reported by Laboratory 2, the lower H/L ratio of 2.8 indicates higher precision in mysid chronic toxicity testing among laboratories--higher even than reported by Schimmel (1981) for an interlaboratory comparison of acute toxicity tests with the same species and chemical. These results suggest that precision can best be maintained if contract laboratories are required to adhere stringently to the standard methods outlined for the mysid life cycle toxicity test using M. bahia.

Comparison of chronic toxicity values for the two chemicals (Tables 2 and 3) suggests that mysids are more sensitive to chronic exposure to the pesticide endosulfan than to the heavy metal silver. As might be expected, variation among laboratories in final chronic toxicity values was greater for the less toxic chemicals. The apparent difference in toxicity of these two chemicals is not surprising, considering the close phylogenetic relationship between the crustacean mysid and the insect pests for which insecticides like endosulfan are developed to control.

Half of the laboratories involved in this project reported adverse effects of the carrier, triethylene gylcol (TEG), used in tests with endosulfan. Survival of TEG control mysids from Laboratories 2 and 6 was 47% and 35%, respectively, compared to survival of seawater control mysids of 87% and 88%. Laboratory 5 reported diminished reproductive success of TEG control mysids compared to mysids of the seawater control. Additive stress accompanying TEG exposure might have been a contributing factor in both the increased lethality associated with endosulfan exposure, as compared with silver exposure, and the reduced interlaboratory variation in chronic toxicity values for endosulfan, as

compared with those for silver. To eliminate any stress on mysids with TEG exposure and possible additive effects of TEG and pesticide exposure, it is recommended that definitive studies be performed to determine what concentrations of TEG affect survival and reproduction of Mysidopsis bahia in a life-cycle exposure study. From the results of this study, maximum TEG concentrations could be determined for further life-cycle toxicity tests with this species.

Examination of MATC limits reported by laboratories provides some indication of precision among test results that might be obtained from a contract laboratory. However, to ensure the most objective comparison of MATC values as they relate to the toxicity test method, the raw data from each laboratory were analyzed by the same statistical procedures. MATC limits and MATC geometric means for silver, based on the same statistical analysis of raw data produced by six different laboratories using the life cycle toxicity test with M. bahia, are presented on Table 4. The mean of the MATC geometric means for silver developed from the data of six laboratories is 40 µg/ℓ with a standard deviation of 31 and a 0.78 coefficient of variation. The lowest concentration of silver producing a significant effect was 15 µg/ℓ and the highest was greater than 140 µg/ℓ, resulting in a H/L ratio of greater than 9.3. This ratio and the coefficient of variation are lower than those calculated from the chronic toxicity values reported by the six laboratories. Nevertheless, the H/L ratio is still higher than that reported by Schimmel (1981) for acute toxicity tests using the same species and chemical and still implies a relatively higher degree of variation between laboratories for mysid chronic toxicity testing than for mysid acute toxicity testing.

An analysis of the raw data from Laboratory 6 revealed that no exposure concentration of silver produced a significant effect on chronic mortality or

Table 4. MATC limits, MATC geometric means, and effect criteria for silver (µg/ℓ) in life cycle toxicity tests with Mysidopsis bahia performed by six laboratories when the raw data of all were analyzed by the same statistical test.

Laboratory	MATC Limits (µg/ℓ)	MATC Geometric Mean (µg/ℓ)	Effect Criterion
1	> 9, <25	15[a]	Reproduction
2	>32, <108	59	Chronic Mortality
3	>20, <30	24	Reproduction
4	>14, <19	16[a]	Chronic Mortality
5	>66, <110	85[a]	Chronic Mortality/ Reproduction
6	>140	---	Chronic Mortality
mean		40	
standard deviation		31	
coefficient of variation		0.78	

a- same value as reported by laboratory

reproductive success of mysids. Close examination of the raw data from Laboratory 6 shows a high degree of variation within each exposure concentration. For example, the range of survival observations varied from 100% to 75% for control mysids and from 100% to 20% for each of the five exposure concentrations. This large variation within treatments prevented any concentration from being statistically different from the control. Similarly, examination of the survival data within each laboratory showed that standard deviations in survival-percentage data varied from 15% to 26% among the different laboratories. Since the survival percentages in the mysid life-cycle toxicity tests are based on only 5 individuals per replicate retention chamber, the death of only one mysid carries a weight of 20%. An increase in the number of mysids per replicate would allow each mysid death to have a lower percentage value and potentially reduce the variation in survival data within each exposure concentration. Therefore, it is recommended that more than 5 mysids be isolated per replicate within larger retention chambers in the mysid life cycle toxicity test.

For the vast majority of pesticides examined in a life-cycle toxicity test with the estuarine mysid, Mysidopsis bahia, a sublethal reduction in the reproductive potential proved to be a more sensitive criterion for chronic biological effects than direct lethality (Nimmo et al. 1977, 1979, 1980, 1981; McKenney et al., unpublished data). Reproductive impairment during sublethal exposure to pesticides and the accompanying diminished size of mysid populations could have a direct impact on commercially important fish that utilize mysids as food and disrupt the delicate balance in estuarine and marine food webs.

Examination of the data presented by the six laboratories suggested that reproductive success of mysids exposed to endosulfan was not more sensitive to

exposure than chronic mortality (Table 5). Nevertheless, regression analysis of the reproductive data revealed a linear relationship between number of young released per female and endosulfan concentrations. Two laboratories reported significant mortality among control mysids exposed to TEG concentrations far below acceptable concentrations of 5 mg/ℓ, suggesting that the health of mysids during the 28-day testing period might be suspect. Reproduction of unhealty control mysids could be impaired and any further disruption by sublethal endosulfan exposure would be more difficult to discern.

Reproduction of mysids involves multiple release of successive broods of juveniles. The number of young released per female is more variable between broods than within broods (McKenney and Ashton, unpublished data). Variability in the reproductive success of mysids that exists in a short period of time could be determined with more precision if the response were based on a biologicaly significant point in the reproductive process of mysids, e.g., after one brood release, rather than at some arbitrary time, such as after 28 days.

Since fixed treatment levels of the variable, toxicant concentration, were not required among laboratories, multiple covariate regression analysis can best analyze the interlaboratory variation that exists in the lethal and reproductive responses of mysids from life cycle toxicity tests performed by different laboratories. The analysis of the raw data provided by the six different laboratories showed that survival and reproductive success of Mysidopsis bahia was linearly related to endosulfan and silver exposure concentrations, but that these relationships were significantly influenced by the laboratory that produced the data (Table 6 and 7) and this between laboratory variation invalidates further comparison.

Even with these significant differences among the data produced by different laboratories, however, the H/L ratios for chronic toxicity values from

Table 5. MATC limits, MATC geometric means, and effect criteria for endosulfan (µg/ℓ) in the life cycle toxicity test with Mysidopsis bahia performed by six laboratories when the raw data of all were analyzed by the same statical test.

Laboratory	MATC Limits (µg/ℓ)	MATC Geometric Mean (µg/ℓ)	Effect Criterion
1	>0.33, <0.71	0.48[a]	Chronic Mortality/ Reproduction
2	>0.52, <1.12	0.76	Chronic Mortality
3	>0.14, <0.31	0.21	Chronic Mortality
4	>0.28, <0.60	0.41[a]	Chronic Mortality
5	>0.22, <0.28	0.25	Chronic Mortality
6	<0.43	--	Reproduction
mean		0.42	
standard deviaiton		0.22	
coefficient of variation		0.52	

a- same value as reported by laboratory

Table 6. Multiple covariate regression analysis of raw data produced by six laboratories on the effects of silver on survival and reproduction of Mysidopsis bahia in the life cycle toxicity test.

A) Survival

Source	Degrees of Freedom	Sum of Squares	F-value	Probability of larger F-value
Corrected Total	276	283840.74		
Model	11	159408.93	30.86	0.0001
Laboratory (LAB)	5	18669.78	7.96	0.0001
Silver Concentration (SCONC)	1	40186.00	85.58	0.0001
LAB x SCONC	5	42315.63	18.02	0.0001
Error	265	124431.81		

B) Reproductive Success

Source	Degrees of Freedom	Sum of Squares	F-value	Probability of larger F-value
Corrected Total	272	14448.78		
Model	11	7773.89	27.63	0.0001
Laboratory (LAB)	5	5754.50	45.00	0.0001
Silver Concentration (SCONC)	1	1086.68	42.49	0.0001
LAB x SCONC	5	1210.36	9.47	0.0001
Error	261	6674.88		

Table 7. Multiple covariate regression analysis of raw data produced by six laboratories on the effects of endosulfan on survival and reproduction of Mysidopsis bahia in the life cycle toxicity test.

A) Survival

Source	Degrees of Freedom	Sum of Squares	F-value	Probability of larger F-value
Corrected Total	264	321619.85		
Model	11	206538.57	41.28	0.0001
Laboratory (LAB)	5	44142.45	19.41	0.0001
Endosulfan Concentration (ECONC)	1	40764.02	89.62	0.0001
LAB x ECONC	5	15157.14	6.66	0.0001
Error	253	115081.28		

B) Reproductive Success

Source	Degrees of Freedom	Sum of Squares	F-value	Probability of larger F-value
Corrected Total	259	6260.982		
Model	11	2975.773	20.42	0.0001
Laboratory (LAB)	5	1697.983	25.64	0.0001
Endosulfan Concentration (ECONC)	1	437.194	33.00	0.0001
LAB x ECONC	5	596.855	9.01	0.0001
Error	248	3285.209		

different laboratories were as low for the more toxic chemical, endosulfan, as were reported in the interlaboratory comparisons of acute toxicity values using the same species and chemical (Schimmel, 1981). This suggests that the precision of data obtained from life cycle toxicity tests using mysids as performed by different laboratories is relatively better for the more toxic chemicals. The H/L ratio becomes smaller for silver, the less toxic chemical, when laboratories that do not stringently follow the outlined methods are excluded and the same statistical test is used to analyze the data from laboratories. Furthermore, by modifying the experimental design as recommended in this report, even greater precision may be obtained in chronic toxicity values for the life cycle toxicity test with Mysidopsis bahia.

RECOMMENDATIONS

(1) Should future interlaboratory comparisons of toxicity tests be considered, a more exact expression of program office needs would be desirable. For example, if the intent of the interlaboratory comparisons was solely to provide information on the precision of the chronic mysid toxicity test, then selection of participating laboratories should be more dependent on the competency of the laboratory to perform the test than on the cost of performing the test. Laboratories chosen for this project were selected equally for their competency with the test method and for their ability to perform the test at a low cost. While the selection process in this instance more closely mimics industrial considerations for selection of performing laboratories, it does so at the expense of limiting the utility of the data for estimating variability in the test method.

(2) Variability in the mysid chronic toxicity test could have been more precisely determined if all laboratories had performed the chronic test with the same fixed exposure concentrations. Furthermore, the performance

of replicate tests by each laboratory would have provided an estimate of variability within laboratories. The use of fixed treatments between laboratories and replicate tests within laboratories would have allowed the results to be statistically analyzed by an analysis of variance and a more accurate measure of variance associated with the test could have been determined.

(3) To reduce variation between laboratories participating in the interlaboratory comparison, it is imperative that all laboratories stringently adhere to the outlined method.

(4) Future tests should consider using a larger number of test animals per container; such a practice would decrease the influence of mortality percentages in a small test population.

(5) To reduce variation in the reproductive responses of mysids as a result of sublethal toxicant exposure, it is recommended that the life-cycle toxicity test using Mysidopsis bahia be terminated after release of the first brood rather than after 28 days.

LITERATURE CITED

Bahner, L.H., C.D. Craft, and D.R. Nimmo. 1975. A saltwater flow-through bioassay method with controlled temperature and salinity. Progressive Fish-Culturist 37:126-129.

Box, G.E.P., W.G. Hunter, and J.S. Hunter. 1978. Statistics for Experimenters, New York: John Wiley and Sons.

Draper, N.R. and H. Smith. 1981. Applied Regression Analysis, New York: John Wiley and Sons.

Mount, D.I. and W. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Research 2:21-29.

Nimmo, D.R., L.H. Bahner, R.A. Rigby, J.M. Sheppard, and A.J. Wilson, Jr. 1977. Mysidopsis bahia: An estuarine species suitable for life-cycle toxicity tests to determine the effects of a pollutant. In Aquatic Toxicology and Hazard Evaluation, pp. 109-116, ed. by F.L. Mayer and J.L. Hamelink. Philadelphia: American Society for Testing and Materials.

Nimmo, D.R., R.A. Rigby, L.H. Bahner, and J.M. Sheppard. 1978a. Acute and chronic effects of cadmium on the estuarine mysid, Mysidopsis bahia. Bull. Environ. Contam. Toxicol. 19(1):80-85.

Nimmo, D.R., T.L. Hamaker, and C.A. Sommers. 1978b. Entire life cycle toxicity testing using mysids (Mysidopsis bahia) in flowing seawater. In Bioassay Procedures for the Ocean Disposal Permit Program. EPA-600/9-78-010, U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida. pp. 64-68.

Nimmo, D.R., T.L. Hamaker, J.C. Moore, and C.A. Sommers. 1979. Effect of diflubenzuron on an estuarine crustacean. Bull. Environ. Contam. Toxicol. 22:767-770.

Nimmo, D.R., T.L. Hamaker, J.C. Moore, and R.A. Wood. 1980. Acute and chronic effects of Dimilin on survival and reproduction of Mysidopsis bahia. In Aquatic Toxicology, pp. 366-376, ed. by J.G. Eaton, P.R. Parrish, and A.C. Hendricks. Philadelphia: American Socity for Testing and Materials.

Nimmo, D.R., T.L. Hamaker, E. Matthews, and J.C. Moore, 1981. Overview of the acute and chronic effects of first and second generation pesticides on an estuarine mysid. In Biological Monitoring of Marine Pollutants, pp. 3-19, ed. by F.J. Vernberg, A. Calabrese, F.P. Thurberg, and W.B. Vernberg. New York: Academic Press.

Schimmel, S.C. 1981. Results: Interlaboratory Comparison--Acute Toxicity Tests Using Estuarine Animals. EPA-600/4-81-003, U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, FL. 14 p.

Sokal, R.R. and F.J. Rohlf. 1969. Biometry, San Francisco: W.H. Freeman and Co.

Sprague, J.B. 1969. Review paper: Measurement of toxicity in fish. 1. Bioassay methods for acute toxicity. Water Research 3(11):793-821.

Steel, R.G.D. and J.H. Torrie. 1960. Principles and Procedures in Statistics, New York: McGraw-Hill.

USDA Agricultural Research Service. 1977. Comparisons among treatment means in an analysis of variance. (Copies available from: Data Systems Aplication Division, Agricultural Research Service, U.S.D.A., Room 013, NAL Building, Beltsville, Maryland 20705).

Appendix

Attachment 1

Scope of Work

Performance requirements of the contract consist of one 28-day entire life cycle toxicity test using mysids (Mysidopsis bahia) with silver nitrate and one test using the insecticide endosulfan. Project Officer will supply the test compounds (silver nitrate and endosulfan). Contractor must provide his own mysids for experimental purposes; guidance on their culture is provided in "Bioassay Procedures for the Ocean Disposal Permit Program" (EPA-600/9-78-010). Contractor must provide a sample of 10 adult Mysidopsis bahia and description of their source to the Project Officer to verify identification of the species prior to its use in the tests. Test procedures to be followed are Attachment 2 of this document and are a modification of those in "Bioassay Procedures for the Ocean Disposal Permit Program".

Every effort should be made to follow the bioassay procedures attached to this document ("Entire Life Cycle Toxicity Test Using Mysidopsis Bahia in Flowing Seawater"). Consideration will be allowed for well-documented deviations from these procedures if such are requested as part of the bid. If none are requested, none will be considered after contracts are awarded.

Attachment 2

Entire Life Cycle Toxicity Test Using Mysidopsis bahia in Flowing Seawater

Introduction

The purpose of this method, based on Nimmo et al. (1978), is to determine effects of continuous exposure of a pollutant on the survival and reproduction of Mysidopsis bahia through a life-cycle. Modifications of this method have been used to determine chronic toxicities of metal and pesticide (Nimmo et al., 1977).

Physical Systems

Test Water--

1. The source of test water must be a natural water with a salinity $\geq$ 15 $^{o}/_{oo}$ but $\leq$ 30 $^{o}/_{oo}$. Deionized water must be used to dilute water if > 30 $^{o}/_{oo}$.

2. Seawater must be filtered to remove particles 5 µm or larger and to remove planktonic larvae that grow, then prey on mysids or their food during the test.

3. The water source must be analyzed for pollutants such as pesticides, PCB's, and particularly for those chemicals being investigated in the toxicity test. Water must meet the specifications for Alternative Dilution Waters as given in Committee on Methods for Toxicity Tests with Aquatic Organisms (1975).

4. Test water must not come in contact with any material (such as metal, plexiglass, or plastic) not considered inert.

Dosing apparatus--

All tests must be conducted in intermittent flows from a diluter (Mount and Brungs, 1967) or in continuous flow with the toxicant added by an infusion pump.

Aquaria--

Glass aquaria should be approximately 30 cm x 60 cm x 18 cm; each large enough to hold eight retention chambers described below. A self-starting siphon in each aquarium fluctuates the water depth between 2 and 8 cm.

Mysid Retention Chambers--

The chambers consist of a standard, 10-cm glass Petri dish (or cover) to

which a 15-cm-high cylinder of Nitex[R] screen (mesh number 210) is attached by silicone cement.

Test Procedures

Flow Rate of Test Water--

Flow rates to each aquarium must (1) provide no less than 90 % replacement in 8 to 12 hours (Sprague, 1969); (2) maintain dissolved oxygen > 60 % saturation; and (3) maintain the toxicant concentration. Our flow rate is 25ℓ/hour/test aquarium for the continuous-flow system; for the diluter, 6ℓ/hour/test aquarium. Dissolved oxygen determinations must be made once weekly for each aquarium. The pH of the test water must be determined at the start and end of test.

Lighting--

Lighting is continuous, using 40-watt cool-white fluorescent tubes.

Temperature--

Test temperatures should be maintained at 25° ± 2°C.

Cleaning and Aeration--

Test chambers used to retain the mysids are not cleaned during the test; instead, mysids are pipetted to pre-cleaned retention chambers. For aeration, a small, gentle stream of compressed air is delivered into each chamber to safeguard against possible anoxic conditions and to create a current that aids mysid orientation and feeding.

Food--

All mysids in the retaining chambers are fed 48-hour-old Artemia nauplii

---

[R]Nitex is a registered trademark of Tobler, Ernst and Trabor, Inc., Murray St., New York, NY. Reference to commercial products does not constitute endorsement by the Environmental Protection Agency.

daily. Food must alwasy be available to the mysids but not in excessive amounts.

Food requirements increase as juvenile mysids mature. Food must be analyzed and must not be used if the total concentration of organochlorine pesticides plus PCB's exceeds 0.3 µg/g (wet weight).

Concentrations of Toxicants--

1. A minimum of five concentrations of the toxicant and a control with carrier are used. A control without carrier need not be used if triethylene glycol or distilled water alone is used as the carrier. Concentration of triethylene glycol in water must not exceed 5 mg/ℓ. Toxicant selection must include at least one concentration which significantly effects survival or reproduction and one that does not. Concentrations for these chronic toxicity tests must be based on results of 96-hour acute static or flow-through toxicity tests. Guidance for acute toxicity testing can be found in Borthwick (1978) and Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). Selection of test concentrations is difficult because chronic effects on survival or reproduction of mysids can occur at concentrations that range from 0.5 to 0.0001 of the 96-hour LC50. The accuracy of the selection process can be improved by some preliminary acute 96-hour tests using both adults and juveniles and by acute tests to determine the incipient LC50 (Sprague, 1969). Often a satisfactory spectrum of concentrations consists of the mysid 96-hour LC50 value as the highest concentration of the toxicant and a dilution factor of 0.5 for the four lower concentrations.

2. The carrier for silver nitrate must be glass-distilled water, and the carrier for endosulfan must be triethylene glycol.

3. One water sample from each aquarium must be analyzed weekly during the life-cycle test for toxicant concentration. If some concentrations are not

detectable analytically and need to be nominal, the detetable concentrations and stock used to prepare the non-detectable concentrations must be measured.

Starting the test--

At the onset, 250 to 350 gravid female mysids are isolated in a 5-liter glass battery jar. An extremely slow flow of salt water (about 4 drops/second) at salinity $\geq$15 but $\leq 30^{o}/oo$ is allowed to drip into the jar. Water exits through a Nitex collar (mesh number 210) attached to the top of the battery jar. The mysids are given a constant supply of 48-hour-old Artemia nauplii. Juvenile mysids are removed during a period not to exceed 48 hours until their number is sufficient to begin a test. The test is begun with eight retention chambers containing five juveniles each (40 animals) per concentration and the control.

Test Progression--

1. In monitoring daily changes in mysid populations, the retaining chamber is lifted gently from the aquarium, water is drained from the Nitex cylinder to the level of the Petri dish, and the chamber is placed on a lighted counter table.

2. Throughout the duration of the test, survival and mortality are noted and recorded daily; dead mysids are removed. A mortality greater than 25 % among the 40 mysids initially introduced into the control invalidates the test.

3. On days 10 to 12, or when sex is readily discernible, the number of males and females (identified by development of brood pouches) in each retention chamber are recorded.

4. Initial release of progeny usually occurs between day 13 and 16 of the test. Progeny are counted, recorded, and removed daily.

5. The life-cycle test must continue for 28 days, which allows the females tested to complete multiple broods.

Statistical Analysis

A chronic toxicity value must be calculated as the geometric mean of the MATC (maximum acceptable toxicant concentration) limits obtained from the most sensitive criterion for effect in the life-cycle test. The MATC limits are the range of concentration between the concentration that has no significant effects on any portion of the life cycle and the lowest concentration that has significant effects. Analysis of variance and an appropriate post hoc test (Student-Newman-Keuls, Duncan's, Dunnett's, etc.) must be conducted to compare survival or reproductive success for multiple treatments and control (USDA Agricultural Research Service, 1977). For each retention chamber, reproductive success (number of progeny per female) is determined by dividing the number of progeny produced by the maximum number of females identified in that chamber. Levels of significance for all statistical analyses must be $\alpha \leq 0.05$.

References

Borthwick, P.W. 1978. Methods for acute static toxicity tests with mysid shrimp (Mysidopsis bahia). In: Bioassay Procedures for the Ocean. Disposal Program, U.S. Environmental Protection Agency, EPA-600/9-78-010: 61-63.

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. U.S. Environmental Protection Agency, EPA-660/3-75-009. pp. 1-61.

Mount, Donald I., and William Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Research 2:21-29.

Nimmo, D.R., L.H. Bahner, R.A. Rigby, J.M. Sheppard, and A.J. Wilson, Jr. 1977. Mysidopsis bahia: an estuarine species suitable for life-cycle toxicity tests to determine the effect of a pollutant. F.L. Mayer and J.L. Hamelink, Eds., American Society for Testing and Materials STP 634: 109-116.

Nimmo, D.R., T.L. Hanmaker, and C.A. Sommers. 1978. Entire life cycle toxicity test using mysids (Mysidopsis bahia) in flowing water. In: Bioassay Procedures for the Ocean Disposal Program. U.S. Environmental Protection Agency, EPA-600/9-78-010. pp. 64-68.

Sprague, J.B. 1969. Review Paper: Measurement of toxicity to fish. 1. bioassay methods for acute toxicity. Water Research 3(11):793-821.

U.S.D.A. Agricultural Research Service, 1977. Comparisons Among Treatment Means in an Analysis of Variance (Copies available from Data Systems Application Division, Agricultural Research Service, USDA, Room 013, NAL Building, Beltsville, Maryland 20705).

Attachment 3

Final Report

A final report must include the following:

A. Name of test, investigator, laboratory, and dates of test.

B. Information on test water:

1. Source of Water

2. Filtering information

3. Reports of chemical analyses for contaminant concentrations

4. Salinity (ppt; range and mean)

5. Temperature (^{o}C; range and mean)

6. pH at start and end of test

7. Dissolved oxygen determinations during test (ppm)

C. Description of toxicant delivery system:

1. If metering pumps are used--

a. Pump manufacturer and pump type

b. Pump infusion rate (mℓ/hour) and water delivery rate (ℓ/hour)

2. If diluter is used--

a. Type (solenoid, siphon) and brief description of operation

b. Delivery rate (ℓ/hour)

D. Information on test aquaria and mysid retention chambers:

1. Size (cm) and materials used

2. Lighting for the test

E. Source of mysid brood stock and age of test animals:

1. If collected--

a. Location and dates

b. Salinity, temperature, and acclimation history

2. If purchased--

a. Supplier's name and address

3. If cultured in contractor's laboratory--

a. Original collection location and date

b. Pertinent history of cultured mysids

4. Age range of juvenile mysids used to begin the test (within how many hours of age).

F. Food:

1. Source of Artemia (manufacturer)

2. Analysis report on contaminant concentrations

G. Carrier (solvent):

1. Type, manufacturer, and purity.

2. Concentration used in test water (nominal)

H. Controls and toxicant concentrations

1. Type of controls and the toxicant concentrations tested

2. Mean and range of toxicant concentrations (µg/ℓ) for each test treatment and control measured

3. Copies of chemical analysis reports.

I. Test raw data:

1. Daily counts of mysids by--

a. Number alive and number dead in each replicate of each treatment and control

b. Number of each sex (when discernible) in each replicate of treatment and control

c. Number of progeny (after onset of reproduction) in each replicate of each treatment and control.

J. Summary of test data:

1. Total counts of mysids at the end of the 28-day test by:

   a. Number alive and number dead in each replicate of each treatment and control

   b. Maximum number by sex in each replicate of each treatment and control

   c. Number of progeny in each replicate of each treatment and control

   d. General observations on other effects or symptoms and on anything unusual about the test.

K. Statistical analysis report:

1. If computer, copy of printout results

2. If calculator, computations of analysis (pertinent calculations).

L. Detailed discussion of results pertaining to statistical analysis of survival success data and including resulting MATC limits (maximum acceptable toxicant concentration) and a chronic toxicity value (geometric mean of the MATC) for each toxicant.

Appendix Table 1. Results from six laboratories of chronic toxicity tests with *Mysidopsis bahia* for silver.

Laboratory	Measured Silver Concentration (µg/ℓ)	Survival Percentage after 28 Days	Young Released per Female after 28 days
1	Seawater Control	91	8.88
	9	95	9.75
	25	92	3.29[a]
	36	98	2.41[a]
	64	90	1.02[a]
	138	25[a]	0.00[a]
2	Seawater Control	97	0.75
	3	97	0.26
	11	85	1.14
	32	90	0.53
	108	82[a]	0.00
3	Seawater Control	82	3.31
	8	72	4.21
	20	65	2.50
	30	62	2.08
	93	88	0.00[a]
	166	72	0.00[a]
4	Seawater Control	92	3.71
	14	92	3.98
	19	70[a]	3.73
	31	28[a]	2.94
	70	28[a]	0.00[a]
	191	0[a]	0.00[a]

Appendix Table 1. (Continued)

Laboratory	Measured Silver Concentration (µg/ℓ)	Survival Percentage after 28 Days	Young Released per Female after 28 day
5	Seawater Control	78	11.09
	9	80	24.02
	16	75	17.02
	33	72	18.08
	60	65	14.02
	110	20[a]	0.00[a]
	197	0[a]	0.00[a]
6	Seawater Control	92	1.90
	9	60	3.21
	20	70	3.63
	32	70[a]	4.25
	66	75	0.25
	140	72	0.00

[a]significantly lower than the control ($\alpha = 0.05$)

Appendix Table 2. Results from six laboratories of chronic toxicity tests with Mysidopsis bahia for endosulfan.

Laboratory	Measured Endosulfan Concentration (µg/ℓ)	Survival Percentage after 28 Days	Young Released per Female after 28 days
1	TEG Control	88	15.14
	0.08	95	8.21
	0.20	90	7.19[a]
	0.33	88	10.10
	0.71	67[a]	4.59[a]
	1.18	60[a]	3.65[a]
2	TEG Control	63	0.22
	0.07	97	2.45
	0.19	70	1.79
	0.52	46	0.00
	1.12	17[a]	0.90
3	TEG Control	80	4.53
	0.06	70	4.81
	0.14	65	3.95
	0.31	50[a]	3.61
	0.42	55[a]	0.00[a]
	0.51	45[a]	0.55[a]
4	TEG Control	92	2.94
	0.28	92	2.61
	0.60	65[a]	2.65
	1.26	18[a]	0.38[a]
	2.42	2[a]	0.00[a]
	4.87	0a	0.00a

Appendix Table 2. (Continued)

Laboratory	Measured Endosulfan Concentration (µg/ℓ)	Survival Percentage after 28 Days	Young Released per Female after 28 day
5	TEG Control	92	9.44
	0.12	80	8.72
	0.22	90	8.10
	0.28	70[a]	4.29[a]
	0.58	50[a]	7.88
	1.71	25[a]	2.19[a]
	2.77	2[a]	0.00[a]
6.	TEG Control	35	2.56
	0.43	24	0.56[a]
	0.57	30	0.06[a]
	1.60	18	0.31[a]

[a] significantly less than the control ($\alpha = 0.05$)

10604 3

3736

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL RESEARCH LABORATORY
SABINE ISLAND
GULF BREEZE FLORIDA 32561

February 26, 1982

SUBJECT: Apparent Minor Errors in Data Cited in Your Report on Acute Toxicity Test Interlaboratory Variation and Our Annual Report

FROM: David J. Hansen
Research Aquatic Biologist
Experimental Environments Branch

TO: Steven C. Schimmel
ERL/Narr

Our branch has prepared an annual report which is being reviewed within our laboratory. As a part of this report, I prepared a summary of all round-robin results. Therefore, the acute and bioconcentration test results were summarized and your name listed as the first author. I am sending you this report to insure that I have not misrepresented your findings. Please call me if you identify errors or to confirm acceptability of this brief summary.

I, also, asked Jim Patrick and Jim Clark to confirm the data. In doing so, they identified certain computational errors in your report that I felt you should know about. The average LC50 from some endosulfan nominal concentration tests are not exact; i.e., 0.24 vs. 0.221, 0.84 vs. 0.804, 1.02 vs. 1.0116, and 2.41 vs. 2.405. For the annual report, we felt that the data in your report should be used. However, because your name is on both reports, you should have the option of deciding if the report to OTS and the annual report should be revised.

Attachment

This memo is incomplete - data pertains to RTI 3736 - write to David Hansen for more info.

Analysis of Proper Usage of Acute & Chronic Test Data for Silver and Mysids. Values are in ug/l

D. Hansen 10/23/85

Laboratory	96 hr LC50 (TEST TYPE)	Chronic Value	Acute/Chronic Ratio
PL-N (#1)	✓264 (S,N,Ag)[2]; ~~274 (FT,N,Ag)~~ [1] Lab 2 McKenney series; ✓256 (FT,M)[1]; ~~249 (ET,M)~~ [4]	~~19~~ 23[4] 18.76	✓13.2
RCO (#2) 10/21 - Tim Waiwood will sort out ultimately	✓251 (S,N,AgNO3)[1]; ~~282 (FT,N,AgNO3)~~ [1]; ✓300 (FT,M)[1]		
G+G (#3)	✓203 (S,N,Ag)[1], ~~468 (FT,N,Ag)~~ [1]	16[2]31	~~5.375~~ 2.723
Called 10-16-85 Rod will check - Ag	✓86 (FT,M)[1], ✓300 (S,N,Ag)[2] Lab 3 McKenney series		
E Aquarium (#4) at Parker - Metal	✓248 (S,N,Ag)[1], ~~325 (FT,N,Ag)~~ [1]	✓5[2]	✓62.60
	✓313 (FT,M)[1], ✓298 (S,N,Ag)[2] Lab 6 McKenney series		
N - at Res. Lab (#5) & Manning says Ag 10-16-85	✓178 (S,N,Ag)[1], ~~248 (FT,N,Ag)~~ [1]		
	✓65 (FT,M)[1]		
PL, Gulf Breeze (#6)	✓117 (S,N,AgNO3)[1], ~~211 (FT,N,AgNO3)~~ [1] Lab 1 McKenney series	15✓15[2]	✓8.800
Called Hawk - 10/21 left to return 10/23	✓132 (FT,M)[1], 141 (S,N,AgNO3)[2]		
Jones Edmunds & Assoc	300 (S,N,?)[2] Lab 4 McKenney series	53[2]	—
Battelle NE - Ron Butler + Jeff Cardin Lab Books	✓64 (FT,N,Ag)[2] Lab 5 McKenney series	✓85[2], 88[3]	—

1. Values as reported in S.C. Schimmel (1981). Do not use LC50 values reported as flow-through (FT) - nominal (N) as these are from the same test as FT - measured (M) values.
2. Values as reported in C.L. McKenney (1982). Chronic values are those reported by each laboratory not ones recalculated by McKenney.
3. Value calculated using 70 ug/l as NOEC and 110 ug/l as OEC (Butler et al, 19